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Povidone-Iodine (PVI) has a profound effect on in vitro osteoblast proliferation and metabolic function and inhibits their ability to mineralise and form bone

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Structured Abstract

Study Design. A study examining the clinical protocol of scoliosis wound irrigation, demonstrating Povidone-Iodine's (PVI) effect on human osteoblast cells. Primary and immortal cell line osteoblasts were treated with 0.35% PVI for 3 minutes, and analyzed for proliferation rate, oxidative capacity and mineralisation.

Objective. To model spinal wound irrigation with dilute PVI *in vitro,* in order to investigate the effect of PVI on osteoblast proliferation, metabolism and bone mineralisation.

Summary of Background Data. Previously PVI irrigation has been proposed as a safe and effective practice to avoid bacterial growth following spinal surgery. However, recent evidence in multiple cell types suggests that PVI has a deleterious effect on cellular viability and cellular function.

Methods. Primary and immortal human osteoblast cells were exposed to either PBS control or with 0.35% PVI for 3 min. Cellular proliferation was measured over the duration of 7 days by MTS assay. Oxygen consumption rate (OCR), extracellular acidification rate (ECAR) and proton production rate (PPR) were analysed using a Seahorse XFe24 Bioanalyzer. Protein expression of the electron transport chain subunits CII-SDHB, CIII-UQRCR2 and CV-ATP5A were measured via Western blotting. Mineralised bone nodules were stained with alizarin red.

Results. Expressed as a percentage of normal osteoblast proliferation, osteoblasts exposed to 0.35% PVI exhibited a significant 24% decrease in proliferation after 24 h. This was a sustained response, resulting in a 72% decline in cellular proliferation at 1 week. There was a significant reduction in OCR, ECAR, and PPR ($p<0.05$), in osteoblasts that had been exposed

to 0.35% PVI for 3 min, coupled with a marked reduction in the protein expression of CII-SDHB. Osteoblasts exposed to 0.35% PVI exhibited reduced bone nodule mineralisation compared to control PBS exposed osteoblasts ($p<0.01$).

Conclusion. PVI has a rapid and detrimental effect on human osteoblast cellular proliferation, metabolic function, and bone nodule mineralisation.

Key Words: Povidone-Iodine, PVI, Osteoblasts, wound irrigation, Spinal Surgery, Bone Mineralization, Cellular Metabolism, Cell Viability, Cell Proliferation

Level of Evidence: N/A

Introduction

Surgical wound infection is a serious complication following spinal surgery. Infection rates in spinal surgery vary hugely and are dependent on a plethora of factors including patient co-morbidities and type of surgery being performed. In particular deformity correction procedures, with long wounds, long operating times and medical co-morbidities; infection rates can be as high as 19%. Surgical site infections can progress to deep infection, incur multiple operations with increased healthcare costs and impaired patient outcomes (1-6).

To reduce the risk of wound infections, many spinal surgeons are using intra-operative antimicrobial wound irrigates (7). One such irrigate which has been advocated and utilised is dilute Povidone-Iodine (PVI) (8). However, there is a paucity of evidence supporting this off-label practice. Two clinical papers have previously described this as a safe and effective practice (8, 9) but there is evidence that PVI has a deleterious effect on cellular viability and cellular function. Studies across multiple cells types and tissues, including fibroblasts, keratinocytes, chondrocyte, immune cells and synovial tissue have now demonstrated that PVI has cytotoxic effects (10-13).

The PVI wound irrigation technique, as described by Chang et al. (9), involves complete immersion of the wound for 3 minutes with a preparation of 0.35% of PVI (diluted from commercially available 10% PVI "Videne"), followed by copious irrigation with normal saline, prior to decortication and wound closure.

Two laboratory studies (one human, one animal) describe the effects of PVI on osteoblasts. Whilst results were heterogeneous, all described a toxic effect of PVI on the osteoblast (14, 15).

From a spinal surgery perspective, there are no studies which have examined whether PVI at clinically advocated concentrations has deleterious effects on human osteoblast viability and function. Addressing this is imperative since osteoblast proliferative and metabolic activity, together with their ability to form bone and mineralise, is essential for the establishment of solid fusion post-surgery.

The aim of this study was to model the clinical protocol *in vitro,* in order to investigate the effect of dilute PVI washout on osteoblast proliferation, metabolic function and osteoblast bone nodule formation and mineralisation.

METHODS

3.1 Cell culture

Primary human osteoblast cells were cultured in differentiation media (10% fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100µg/ml), L-glutamine (2mM), non-essential amino acids (1%, Invitrogen) and Amphotericin B (2.5 µg/ml), β-glycerophosphate (2mM), Ascorbic acid (50µg/ml) and Dexamethasome (10nM)) entirely. The human osteoblast cell line hFOB 1.19 (ATCC® CRL-11372™) (ATCC, England, UK) was cultured in growth media (1:1 mixture of Ham's F12 Medium Dulbecco's Modified Eagle's Medium, with 2.5 mM L-glutamine (without phenol red), 10% fetal bovine serum (FBS), and 0.3mg/ml G418 prior to differentiation media.

3.2 Preparation of primary human osteoblasts

Ethical approval was granted by the United Kingdom (UK) National Research Ethics Service (National Health Authority, reference NRES 14-ES-1044), and institutionally approved and sponsored by the University of Birmingham as required under the UK Research Governance

Framework. Study participants were provided in advance with a participant information sheet, and a participant consent form. Following patient's written consent, the femoral head was collected from a female patient (aged 62 years) undergoing total joint replacement surgery for hip osteoarthritis. The articular cartilage was removed from the femoral head and the subchondral bone cut into small chips. The bone chips were then washed thoroughly in serum-free primary osteoblast media to remove any excess blood, connective or adipose tissue and then incubated in differentiation media in a culture flask at 37°C (5% CO_2). Differentiation media was replaced with fresh media 2x per week, and the bone chips removed upon the appearance of osteoblast cells.

3.3 Osteoblast proliferation assay

Primary human osteoblasts and the human osteoblast cell line (hFOB 1.19) were plated at $6x10^3$ cells per well in a 96 well plate. At confluency, the cells were incubated with either PBS control or with 0.35% PVI (diluted in PBS) for 3 minutes at room temperature. The wells were then aspirated, washed 5x with PBS and then placed back into osteoblast growth media. After 24, 48, 120, 144 and 168 h at 33°C, an MTS (Cell Titer Aqueous One Solution Cell Proliferation Assay, Promega) assay was performed as per manufacturer's instructions as a measure of osteoblast proliferation.

3.4 Osteoblast metabolic function

Human osteoblast cells (hFOB 1.19) were plated at $6x10^3$ cells per well in an XF^e 24 Cell Culture Microplate (Seahorse Bioscience, USA). At confluency, the cells were stimulated with 0.35% PVI diluted in PBS or with PBS control for 3min, thoroughly washed as described previously and placed back into growth media. After 24 h at 33°C, XF Assay

medium (XF base medium with 2mM GlutaMAX™) was then added to the plate and incubated at 33°C for 1 h.

Using a XF^e Extracellular Flux Analyzer (Seahorse Bioscience, USA) the oxygen consumption rate (OCR), extracellular acidification rate (ECAR) and proton production rate (PPR) of each well were measured simultaneously, in order to comprehensively profile the metabolic function of the osteoblasts.

3.5 Expression analysis of osteoblast mitochondrial oxidative phosphorylation components

Human osteoblast cells (hFOB 1.19) cells were seeded at 6x10^4 cells per well in a 24-well plate. Cells were stimulated with 0.35% dilute PVI or PBS control for 3min, and then washed in PBS as previously described before being cultured in growth media for 24 h (37°C, 5% CO_2). Following 24 h, cells were washed in PBS and lysed in 1xLaemmli Sample Buffer (2%SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.002% Bromophenol Blue, 0.0625M Tris HCL). Equal amounts of total protein lysates were then applied to 12% SDS PAGE electrophoresis and blotted onto polyvinylidene difluoride (PVDF) membrane at 150V for 1 h. Blots were immunoprobed with an optimised antibody cocktail which contains antibodies to detect key components of mitochondrial oxidative phosphorylation, namely CII-SDHB 30kDa (ab14714), CIII-UQCRC2 (ab14745) and CV-ATP5A (ab14748). Blots were developed using ECL™ Prime Western Blotting Detection Reagent Kit (GE Healthcare, UK) on the ChemiDoc™ Imaging MP System (BIO-RAD, USA).

3.7 Osteoblast bone nodule formation and mineralization assay

Human osteoblast cells (hFOB 1.19) were seeded at $6x10^4$ cells per well in a 24 well plate and treated with or without 0.35% PVI for 3 min as described previously, before being cultured in differentiation media. After 14 days, cells were stained with alizarin red solution in order to quantify the degree of mineralisation following the formation of bone nodules. Briefly, cells were incubated in alizarin red staining solution (0.5% Alizarin Red (Sigma-Aldrich, UK) in 1% ammonia solution at pH 4.5) for 10min at room temperature and washed with PBS to remove excess stain. Cells were then incubated in 10% cetyl pyridinium chloride (Sigma-Aldrich, UK) for 10 min at room temperature. The supernatant was collected from each well and diluted 1:10 with the 10% cetyl pyridinium chloride and read at OD_{550nm} on a SpectraMAX Microplate Reader (Molecular Devices, USA).

3.8 Statistical Analysis

All statistical analyses were carried out using Prism5 (GraphPad Software Inc. California, USA). Unless otherwise stated, all data within figures represents Mean ±SEM. MTS assay results were assessed by a one-way analysis of variance (ANOVA) with a Tukey Post Hoc. OCR, ECAR and PPR, and alizarin red analysis was compared using a Students un-paired t-test.

RESULTS

3.1 Acute exposure to PVI markedly reduces osteoblast proliferation.

Acute 3 min exposure of primary human osteoblasts to PVI at the clinical concentration of 0.35% significantly decreased cellular proliferation at 48, 120 and 168 h post-exposure (Figure 1A). Expressed as a percentage of normal osteoblast proliferation (PBS control), osteoblasts exposed to 0.35% PVI exhibited a 24 ± 3.7% decrease in proliferation after 24 h

(Figure 1B). This was a sustained response, which resulted in a 72% ± 2.3% decline in cellular proliferation at 1 week. The significant effect of PVI on cellular proliferation was also confirmed in the human osteoblast cell line (hFOB 1.19) (Figure 1C & 1D), with a 26 ± 3% after 24 h, and a 42% ± 7% decline in proliferation relative to control after 6 days post-exposure.

3.2 Acute exposure of osteoblasts to dilute PVI ablates osteoblast basal metabolic function.

Assessment of metabolic functional activity of osteoblasts (hFOB.1.19) 24 h post-exposure to 0.35% PVI or PBS control showed a significant reduction in OCR (0.32±0.13 vs -0.05±0.12 pmol/min/μg), ECAR (0.10±0.07 vs 0.01±0.02 mpH/min/μg) and PPR (87.64±18.78 vs 3.33±7.24 pmol/min/μg) (Figure 2A-C) in osteoblasts that had been exposed to 0.35% PVI for 3 min.

3.3 Acute exposure of human osteoblasts to PVI markedly reduces CIII-SDHB protein expression, a key component of oxidative phosphorylation.

To identify if the effect of 0.35% PVI on osteoblast metabolic function was due to a deleterious effect on mitochondrial function we then assessed the protein expression of electron transport chain subunits (II, III and CV), involved in mitochondrial oxidative phosphorylation. Human osteoblasts exposed to 0.35% PVI for 3 min exhibited a marked reduction in protein expression of succinate dehydrogenase B (CII-SDHB), and a moderate reduction in the protein expression of cytochrome b-c1 complex subunit 2 (CIII-UQCRC2), compared to PBS control exposed osteoblasts. The protein expression of CV-ATP5A remained unchanged (Figure 2D).

3.4 Acute stimulation with PVI reduces osteoblast mineralization

We next determined whether the deleterious effects of 0.35% PVI on osteoblast proliferation and metabolic functional activity impacted on the ability of osteoblasts to form bone nodules and mineralise. At confluency, osteoblasts were stimulated with 0.35% PVI for 3 min, washed with PBS thoroughly and allowed to proliferate and differentiate for a further 2 weeks in differentiation media. Alizarin red stained mineralisation was quantified and showed that osteoblasts exposed to 0.35% PVI exhibited significantly reduced bone nodule mineralisation (0.28±0.05), compared to control PBS exposed osteoblasts (0.37±0.06) (Figure 3).

Discussion

PVI is advocated for wound irrigation following spinal surgery (8, 9), despite recent reports of its cytotoxicity in multiple cell types (10-13). Our *in vitro* study demonstrates that dilute PVI has fundamental deleterious effects on human osteoblast cells and their innate ability to mineralise.

Utilising primary human osteoblasts, we have demonstrated that acute exposure of osteoblasts to PVI for only 3 min, followed by thorough washing, is sufficient to profoundly inhibit cellular proliferation. Dilute PVI was found to significantly inhibit proliferation within the first 24 h following the 3 min exposure to PVI. However, perhaps of greater concern, our data also reveals that the negative effects on proliferation are fully sustained over a time-course of 7 days, resulting in a 72% inhibition of proliferation at this time point. This demonstrates that once exposed to dilute PVI the osteoblast cells do not recover their proliferative activity. Critically, we have also demonstrated that exposure to dilute PVI inhibits the innate functional ability of osteoblasts to form mineralised bone nodules.

Our data suggests that exposure to dilute PVI inhibits human osteoblast proliferation and mineralisation by preventing normal osteoblast metabolic function, since both basal oxygen consumption rate (a measure of aerobic mitochondrial respiration; (16)) and extracellular acidification rate and proton production rate (a measure of glycolytic metabolism; (16)) were ablated within 24 h post-exposure to 3 min of dilute PVI.

With regard to previous studies which have reported the effect of PVI on the osteoblast, Kaysinger (1995) found that 5% Betadine (0.5% PVI) was toxic to chicken osteoblasts (15). It is of note that the first non-toxic concentration reported by Kaysinger was 5% Betadine (0.5% PVI). From their results it is therefore not possible to ascertain where between 0.05% and 0.5% the toxic effects of PVI are manifested (15). In the paper of Cheng et al. (8) the concentration of 0.5% PVI appears to have been interpreted as the lowest toxic concentration and hence guided their choice of 0.35% PVI for irrigation. We would argue that this was a fundamental misinterpretation of the report of Kaysinger et al. We have now confirmed that 0.35% PVI is indeed toxic to human osteoblasts.

Cabral et al (2007) assessed PVI exposure in human alveolar bone. They found 10% and 5% PVI resulted in immediate cell death. 1% PVI resulted in rounding up and cellular detachment (implied cell death), and that cellular proliferation was reduced at concentrations of 0.5% PVI and higher. Cabral also reported a significant reduction in Alkaline Phosphatase (AP) production at 0.05% and 0.2% PVI (14), indicative of reduced mineralisation activity. Our findings are in agreement with this, since we also found that PVI reduced osteoblast proliferation and have demonstrated a significant reduction in the production of mineralised bone (as measured by Alizarin red staining) with 0.35% PVI.

Infection rates in spinal surgery vary widely. For example, infection rate following anterior cervical surgery is low, at around 0.1%, but infection rates can be up to 19% in spinal deformity surgery (5, 17). Critically, the results of deep infection can be devastating and occasionally fatal in patient groups with multiple co-morbidities. Clearly therefore, efforts must be taken to reduce spinal infection. A very informative Best Practice Guideline in 2013 made a number of recommendations for the "high risk" spinal surgical patients. These included pre-operative washing with chlorhexidine, nutritional assessment, broad spectrum antibiotics and topical vancomycin powder. It is noteworthy that PVI irrigation was not among the recommendations (18).

In 2005 and 2006 two reports from the same centre described the utilization of 0.35% PVI lavage in spinal surgery (8, 9), and reported a clinically significant reduction in infection rates from 2.9% without PVI lavage, to 0.5% with PVI lavage. Clearly this is a commendable reduction in infection rates apparently attributable to the PVI lavage. However, the goal of many spinal surgeries is the establishment of solid bony fusion (union). Therefore, the potential negative effect of a given irrigate on bone union must be considered alongside any positive benefit on spinal infection rates.

Measurement of union (and non-union) are difficult to comprehensively determine using Computed Tomography (CT) (Carreon et al. 2008). The only reliable method is surgical exploration. In lumbar fusion surgery non-union rates are reported to be 10-20%. In fusions of 4-levels or more, and in particular in adult degenerative scoliosis the rate of non-union can be as high as 20- 60% (9, 19-21).

Our study identifies a potential pathway for impaired bony union following PVI lavage in the clinical setting. The two reports from Cheng et al. (8) clearly state that on radiographic analysis there was no adverse effect on fusion rates. However, it is noteworthy that the patients in these studies were overwhelmingly 2-level or fewer low-back fusions. These particular patients have a fairly predictable union rate of around 80-90%. It is possible therefore that the deleterious effect of PVI on the human osteoblast may have a greater clinical impact on longer fusions with higher inherent risks of non-union. Additionally Chang et al. (9) used plain X-ray for evaluation of bony fusion. This is not a reliable method for assessing bony fusion, with only 68% accuracy in lumbar spine fusions (22).

Outside the scope of this report, but of importance to the surgeon considering using PVI for irrigation of surgical wounds are descriptions of systemic complications following iodine lavage. These include: significant systemic iodine absorption, acute thyroid dysfunction, cardiovascular collapse, renal failure and death. Whilst these are not immediately relevant to bone healing, it does suggest that iodine lavage is not a universally benign phenomenon (23-27).

There are limitations to our study. It is not possible to replicate *in vitro* the entire biological system involved in bone healing. We cannot replicate or ascertain what the effect would be on the pluripotent stem cells that subsequently migrate to the site of bony healing after irrigation. We have only assessed the effect on one cell type (the osteoblast), we do not know what the effect of 0.35% PVI would be on other cell types involved in bone healing such as fibroblasts, osteoclasts and platelets. We also accept that although our primary osteoblasts were human, they were not isolated from spinal bone. It is therefore conceivable that osteoblasts within spinal bone could behave differently to osteoblasts from the appendicular

skeleton. It is reassuring however that our results are similar to those described by Kaysinger from chicken embryonic tibiae (15).

In summary, we have demonstrated a clear deleterious effect of PVI to osteoblast proliferation and function and mineralisation. We suggest the advocated antimicrobial gains may be offset by the potential for impaired bone healing, particularly in long fusion procedures with higher non-union rates, where there is no clinical outcome data relating to PVI irrigation.

Abbreviations

PVI	Povidone-Iodine
PBS	Phosphate buffered saline
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
CII-SDHB	Succinate Dehydrogenase B
CIII-UQCRC2	ubiquinol-cytochrome c reductase core protein II
CV-ATP5A	ATP synthase

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Figure 1. The effect of acute exposure to dilute PVI on the proliferation of primary human osteoblasts and a human osteoblast cell line. **A**. Primary human osteoblasts stimulated with PBS and 0.35% PVI for 3 minutes prior to time dependent MTS assays. ○ = PBS control; ● = 0.35% PVI stimulated (*p<0.05, ***p<0.001). **B**. Percentage proliferation of primary osteoblasts when compared to PBS control stimulated osteoblasts (**p<0.01 between 24 and 120 h, §p<0.001 between 24 and 168 h, †p<0.01 between 48 and 168 h). C. hFOB 1.19 cell proliferation as measured by MTS assay (*p<0.05, ***p<0.001). D. Percentage proliferation of hFOB 1.19 osteoblasts when compared to PBS control stimulated osteoblasts (*p<0.05; n=3).

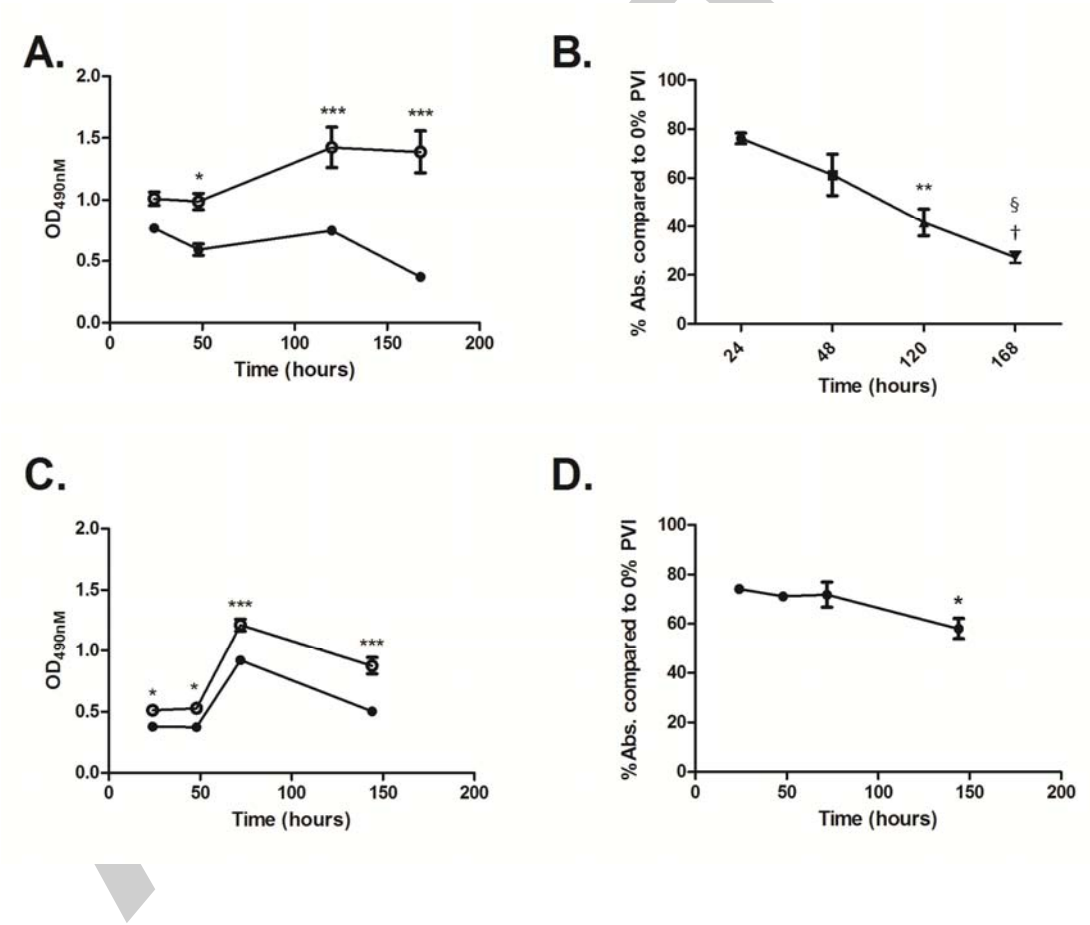

Figure 2. Metabolic activity of stable osteoblast culture following acute PVI stimulation. A. Oxygen consumption rate (OCR) of osteoblasts. B. Extracellular acidification rate of osteoblasts. C. Proton production rate of osteoblasts (Figure 2A-C n=4, *=p<0.05). D. Western blot of cells stimulated with 0.35% PVI or PBS control 24 h prior to lysis and assayed for mitochondrial protein expression (n=3).

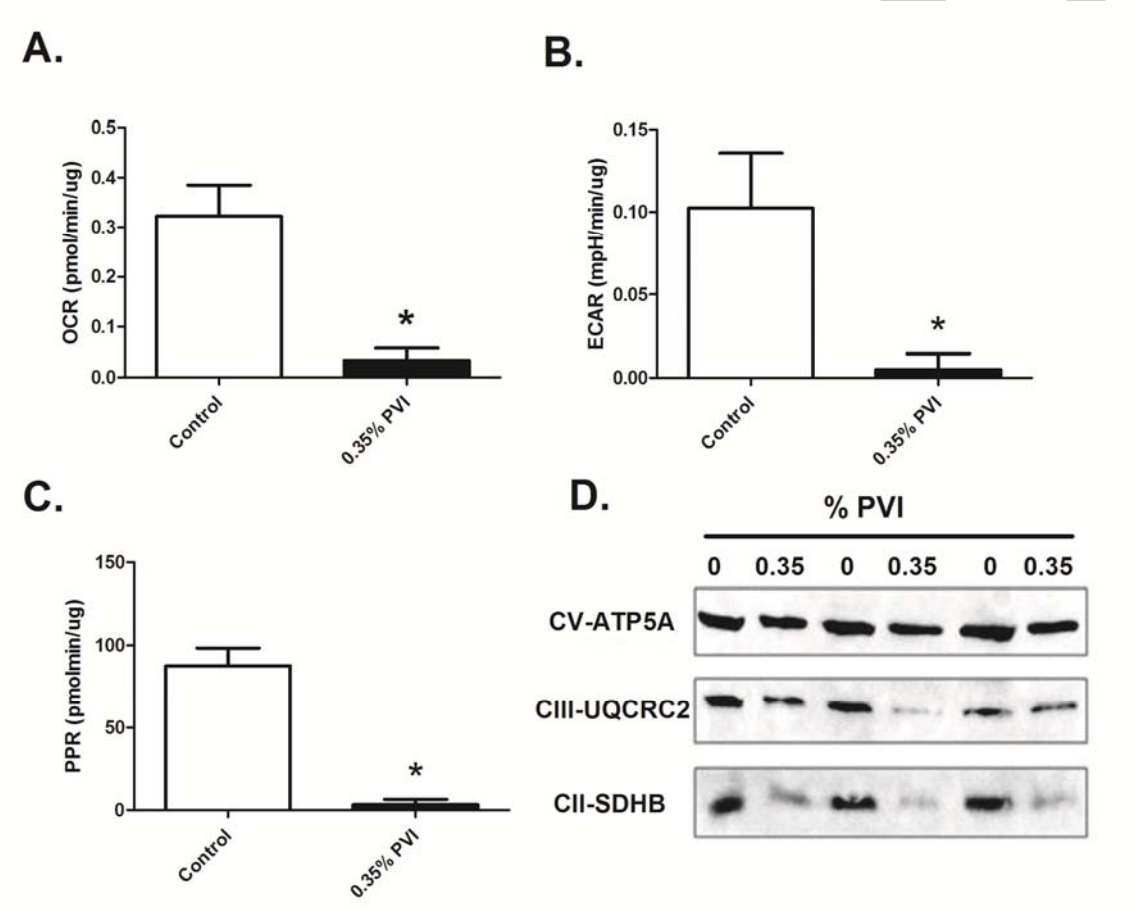

Figure 3. Osteoblast mineralization following acute PVI stimulation. Alizarin red staining of osteoblasts treated with 0.35% PVI or PBS control was quantified at OD_{550nm} using 10% cetyl pyridinium chloride. (n=8, **p<0.01).